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Effects of carbon tetrachloride and azathioprine on diethylnitrosamine and N-2-fluorenylacetamide-induced hyperplastic liver nodule and hepatocellular carcinoma

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Effects of carbon tetrachloride and azathioprine on diethylnitrosamine and N-2-fluorenylacetamide-induced hyperplastic liver nodule and hepatocellular carcinoma*

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Abstract

Effects of carbon tetrachloride (CCl4) and azathioprine (AZP) on the evolution of hyperplastic liver nodules and foci and hepatocellular carcinoma (HCC) were tested in short- and long-term in vivo experiments. In diethylnitrosamine (DEN)-treated rats, which were fed a N-2-fluorenylacetamide (FAA)-containing diet and additionally treated with repeated CCl4 injections, gamma-glutamyl transpeptidase (gamma-GTP)-positive hyperplastic nodules were markedly developed in the 8th week of the experiment. However, their number and area in liver sections were remarkably small in DEN-treated rats fed a diet containing both FAA and AZP. Increased area of gamma-GTP-positive foci was also observed in the 12th week in DEN-injected rats fed a choline-devoid died alone or treated with repeated doses of CCl4 alone. Hepatocellular carcinoma in DEN-injected rats treated with both FAA and CCl4 was first detected in the 21st week, and the incidence up to the 36th week was very high. However, no hepatocellular carcinoma developed in DEN-injected rats treated with both FAA and AZP. The increased activity of liver aniline hydroxylase observed 12 h after the administration of FAA, AZP or DEN alone was not observed when AZP was administered simultaneously with FAA to DEN-injected rats. The mechanisms of the effects of CCl4 and AZP on hepatocarcinogenesis are discussed with special reference to drug interaction.

KEYWORDS: hepatocellular carcinoma, hyperplastic liver nodule, ?-glitamyl transpeptidase, azathioprine, carbon tetrachloride

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EFFECTS OF CARBON TETRACHLORIDE AND AZATHIOPRINE ON DIETHYLNITROSAMINE AND N-2-FLUORENYL-ACETAMIDE-INDUCED HYPERPLASTIC LIVER NODULE AND HEPATOCELLULAR CARCINOMA

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Abstract. Effects of carbon tetrachloride (CCl_4) and azathioprine (AZP) on the evolution of hyperplastic liver nodules and foci and hepatocellular carcinoma (HCC) were tested in short- and long-term *in vivo* experiments. In diethylnitrosamine (DEN)-treated rats, which were fed a N-2-fluorenylacetamide (FAA)-containing diet and additionaly treated with repeated CCl_4 injections, γ-glutamyl transpeptidase (γ-GTP)-positive hyperplastic nodules were markedly developed in the 8th week of the experiment. However, their number and area in liver sections were remarkably small in DEN-treated rats fed a diet containing both FAA and AZP. Increased area of γ-GTP-positive foci was also observed in the 12th week in DEN-injected rats fed a choline-devoid died alone or treated with repeated doses of CCl_4 alone. Hepatocellular carcinoma in DEN-injected rats treated with both FAA and CCl_4 was first detected in the 21st week, and the incidence up to the 36th week was very high. However, no hepatocellular carcinoma developed in DEN-injected rats treated with both FAA and AZP. The increased activity of liver aniline hydroxylase observed 12 h after the administration of FAA, AZP or DEN alone was not observed when AZP was administered simultaneously with FAA to DEN-injected rats. The mechanisms of the effects of CCl_4 and AZP on hepatocarcinogenesis are discussed with special reference to drug interaction.

Key words : hepatocellular carcinoma, hyperplastic liver nodule, γ-glutamyl transpeptidase, azathioprine, carbon tetrachloride.

Various chemicals in our environment, such as aflatoxin B_1 and oral contraceptives, have been proposed as agents involved in hepatocarcinogenesis (1, 2). Nutritional and dietary factors also play a role in the evolution of hepatocellular carcinoma (HCC) (3, 4). Several reports have described tumor growth in man upon treatment with immunosuppresive drugs (5).

The liver is a good target for neoplastic transformation in experimental animals. Experimental hepatocarcinogenesis is considered to be a multistep process consisting of at least two distinct stages, initiation and promotion (6). There have been many analyses of the initiation step, but little is known about the promotion

step. Many investigators have designed experimental protocols for promoting hyperplastic liver nodules and foci, defined as preneoplastic lesions in the liver which progress to HCC, in rats (6-8).

There have been few investigations about the role of liver injury on the process of hepatocarcinogenesis. The author undertook two different, short- and long-term, *in vivo* experiments using the protocols of Shinozuka *et al.* (9) and Tatematsu *et al.* (8) to determine the enhancing and inhibitory effects of the hepatotoxins carbon tetrachloride (CCl_4) and azathioprine (AZP) in the evolution of hyperplastic liver nodules and foci, and HCC. In the short-term experiment, choline-devoid (CD) diet was also used to compare the effects of hepatotoxins with the effects of phenobarbital (PB), a promoter of hepatocarcinogenesis (10). In addition, branched chain amino acid transaminase (BCAA-TA ase, EC 2. 6. 1. 6) isozymes, ornithine decarboxylase (ODC, EC 4. 1. 1. 17) and aniline hydroxylase (AH, EC 1. 14. 1. 1) in the liver were assayed to investigate the mechanisms of the observed effects.

MATERIALS AND METHODS

Animals and chemicals. Male Sprague-Dawley rats (Charles River Japan, Inc., Tokyo), weighing approximately 250 g, were kept for a week on a basal diet (CE-2, Clea Japan, Inc., Tokyo) prior to the experiments. N-2-Fluorenylacetamide (FAA, Nakarai Chemical Co., Kyoto) and AZP (Sigma Chemical Co., St. Louis) were added to the basal diet at a concentration of 0.02 % and 0.05 %, respectively. Diethylnitrosamine (DEN, Wako Pure Chemical Co., Osaka) was dissolved in physiological saline at a concentration of 10 %, and CCl_4 (Nakarai Chemical Co., Kyoto) was diluted with olive oil at a concentration of 50 %. PB (Linasen®, Daiichi Seiyaku Co., Ltd., Tokyo) was added to tap water at a level of 0.05% and administered as drinking water. The animals were given food and water *ad libitum*, and they were fasted for 12 h prior to sacrifice.

Experimental design. The fundamental design of the experiments is shown in Fig. 1. Experiment A, based on a model reported by Tatematsu *et al.* (8), was performed to determine the effect of CCl_4 and AZP on hepatocarcinogenesis after the development of hyperplastic liver nodules in DEN- and FAA-treated rats. Experiment B, after a model designed by Shinozuka *et al.* (9), was performed to test whether similar observations could be obtained without the use of FAA, since FAA itself is known to act as a promoter of hepatocarcinogenesis.

In Experiment A, rats were divided into 3 groups. DEN was injected intraperitoneally at a dose of 200 mg/kg body weight, and the basal diet was given for the initial 2 weeks. The rats were then given a diet containing FAA (Control group), a diet containing FAA and AZP (AZP group), or the diet containing FAA along with subcutaneous injections of CCl_4 at a dose of 0.5 ml/kg body weight twice a week (CCl_4 group). All the rats were subjected to partial hepatectomy at the end of the 3rd week of the experiment. Animals were sacrificed at the 8th week, when hyperplastic liver nodule formation was observed, and also from the 21st to 36th week, when tumor development could be expected.

In Experiment B, rats were given a single intraperitoneal injection of DEN at a dose of 50 mg/kg body weight 18 h after a partial hepatectomy and then placed on the basal diet for 10 days. The control group was continued on the basal diet until sacrifice, and in the other four groups, an AZP containing diet (AZP group), PB solution as drinking water (PB group) or a

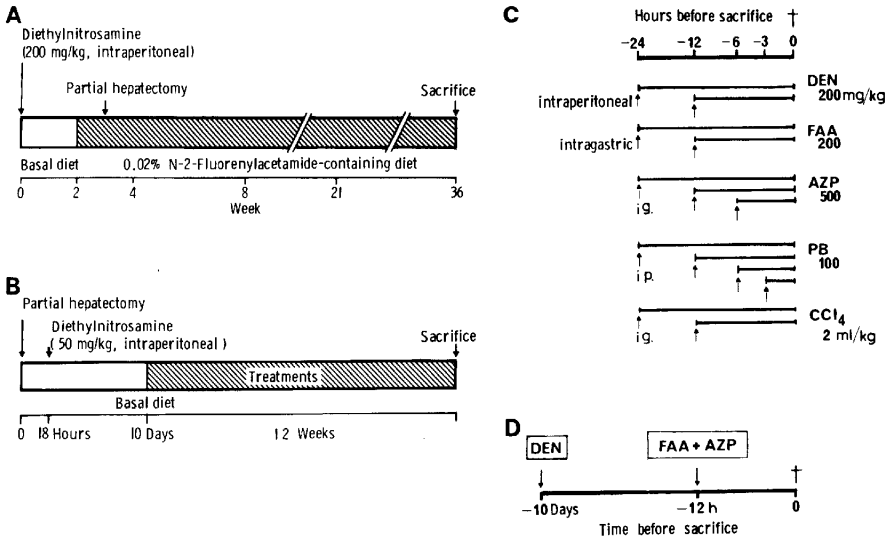

Fig. 1. Schedules of the experiments. Experiment A: Rats were injected with DEN intraperitoneally, and the basal diet was given for 2 weeks (open bar). Then rats were fed on a 0.02 % FAA-containing diet (hatched bar). Partial hepatectomy was performed at the end of the 3rd week. Experiment B: Rats were injected intraperitoneally with DEN 18 h after partial hepatectomy and placed on a basal diet for 10 days (open bar), and then treated with various agents (AZP, PB, CCl_4 and CD) for 12 weeks (hatched bar). Experiment C: Schedule for evaluating the promoting activity of the chemicals used in Experiments A and B. Arrows indicate the time at which a single administration of DEN, FAA, AZP, PB or CCl_4 was performed. ip., intraperitoneal injection. ig., intragastric administration. Experiment D : Schedule for evaluating the mechanism of AZP suppression of hepatocarcinogenesis observed in Experiment A. Other details are given under Materials and Methods.

CD diet (CD group) was initiated, or CCl_4 was subcutaneously injected at a dose of 0.5 ml/kg body weight, twice a week (CCl_4 group). All the animals were killed 12 weeks after the 10th day of partial hepatectomy.

Experiments C and D were carried out to evaluate the mechanisms of the enhancing or inhibitory effects of CCl_4 and AZP on the evolution of hyperplastic liver nodules and HCC observed in Experiments A and B. Experiment C was performed to evaluate the promoting activity of chemicals used in both Experiments A and B. FAA (200 mg/kg body weight) or AZP (500 mg/kg body weight) in 0.5% carboxymethyl cellulose (Nakarai Chemical Co., Kyoto) or CCl_4 (2 ml/kg body weight) in olive oil was intragastrically administered to overnight-fasted rats which were given only water during the treatment. DEN or PB was intraperitoneally injected at a dose of 200 mg/kg body weight and 100 mg/kg body weight, respectively. Animals were sacrificed 3, 6, 12 and 24 h after the administration. Experiment D was designed to evaluate the suppressive effect of AZP on hepatocarcinogenesis induced by DEN and FAA as observed in Experiment A. AZP and FAA were simultaneously administered intragastrically to rats which had been treated 10 days before with an intraperitoneal injection of DEN.

Analytical procedures. BCAA-TAase isozymes, as marker enzymes of hepatocyte differentiation during the process of hepatocarcinogenesis, were assayed using a DEAE-cellulose (Sigma Chemical Co., St. Louis) column as described by Stephen *et al.* (11, 12). The activity of hepatic ODC, a rate-limiting enzyme of polyamine biosynthesis which produces liver hypertrophy and

induces mixed-function oxygenases (13), was determined according to the method of Fukui *et al.* (14). The activity was expressed as pmoles of CO_2 per mg protein per 20 min (14). The activity of liver AH, a microsomal drug-metabolizing enzyme, was measured by the method of Imai *et al.* (15) and expressed as nmoles of product per mg protein (mU). Protein concentrations were determined by the method of Lowry *et al.* (16).

For histochemical examinations, liver tissues were fixed with 99 % cold ethanol and stained with γ-glutamyl transpeptidase (γ-GTP, EC 2. 3. 2. 1) by a modified method of Albert *et al.* (17). The number and area (mm^2) of γ-GTP-positive hyperplastic liver nodules and foci per cm^2 of liver section were quantitatively determined on enlarged microscopic color photograms. The area of hyperplastic liver nodules and foci on the photograms was measured with a Medical Graphics Analyzer (Good Man Co., Ltd., Nagoya).

Serum α-fetoprotein (AFP) levels were measured by radioimmunoassay, and serum glutamate pyruvate transaminase (GPT, EC 2. 6. 1. 2) and γ-GTP activities were determined using a GPT-UV Test (Wako Pure Chemical Co., Osaka) and γ-GTP SET (Iatron Labo. Inc., Tokyo), respectively. The immunosuppressive capability of AZP in Experiment A was evaluated by indirect methods such as analysis of peripheral leukocytes or measurement of spleen weight. All the data were expressed as the mean ± SD. Statistical differences between the mean values were determined by Student's *t* test after analysis of variance.

RESULTS

Experiment A. The body weight of rats throughout the experiment are shown in Fig. 2A. The weights in both the CCl_4 and AZP groups were slightly less than

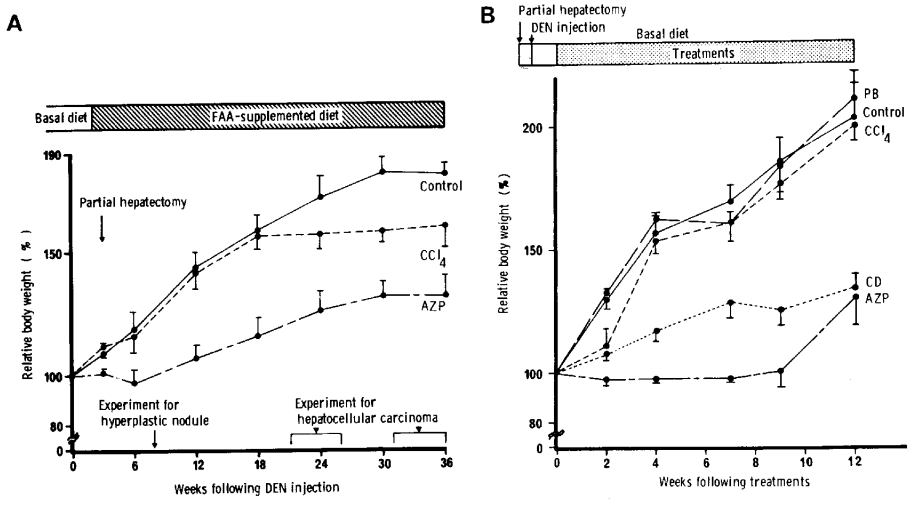

Fig. 2. Changes in body weight during Experiments A and B. (A) Zero time indicates DEN injection, the beginning of the experiment. ●——●, Control group; ●– –●, AZP group, and ●····●, CCl_4 group. (B) Zero time indicates the 10th day of partial hepatectomy. ●——●, Control group; ●– –●, AZP group; ●——●, PB group; ●····●, CCl_4 group, and ●····●, CD group. Relative body weight was calculated by dividing the body weight at the indicated time by that at 0 time of the experiment. Vertical lines indicate standard deviation of the mean.

TABLE 1. SERUM BIOCHEMICAL DATA AND PERCENT LIVER WEIGHT IN DEN- AND FAA-TREATED RATS

Group	Week of the exp.	No. of rats	Liver weight / Body weight (%)	S-GPT (IU)	S-γ-GTP (U)
Control					
HN	[8]	(4)	4.7±0.2	75± 7	26± 3
Non-cancer	[21-36]	(4)	6.1±0.3	139±23	41± 5
Cancer	[31-36]	(2)	7.6	160	140
AZP					
HN	[8]	(4)	3.7±0.2	54± 4	18± 3
Non-cancer	[21-36]	(5)	4.6±0.4	120±41	38±18
CCl_4					
HN	[8]	(3)	6.4±0.1	130±29	69±16
Non-cancer	[31-36]	(1)	7.9	120	86
Cancer	[21-36]	(4)	20.5±1.0	188±39	184±54

HN = Hyperplastic liver nodule
*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

those in the control group. Liver weights of the CCl_4 group in the 8th week were much heavier than in the other groups, and livers in the AZP group were significantly small (Table 1). Hyperplastic liver nodules and foci observed in the 8th week of Experiment A are illustrated in Fig. 3. There were large γ-GTP-positive foci in livers from the control group (Fig. 3A), but much larger hyperplastic liver nodules were observed in the CCl_4 group (Fig. 3C). However, the foci in the AZP group were small (Fig. 3B). The number and area of γ-GTP-positive hyperplastic liver nodules and foci in each group determined at the end of the 8th week are summarized in Table 2. A large hyperplastic liver nodule area was observed in the CCl_4 group. The area was significantly small in the AZP group. Table 2 also shows the number of tumor-bearing rats observed during the 21st to 36th week of the experiment. Hepatic tumors, which were confirmed macroscopically (Fig. 4) or histologically (Fig. 5), were first detected in the 21st week in the CCl_4 group, and the number of rats with hepatic tumors increased as the duration of treatment increased. However, no tumor was detected in the AZP group within 36 weeks. The liver surfaces in the control group had tumors with smooth white capsules (Fig. 4A). Most tumors replaced a major portion of the right lateral lobe especially in the CCl_4 group (Fig. 4C). Livers in the AZP group had mildly granular surfaces without tumors, and the dorsal surfaces were more granular than the ventral areas (Fig. 4B). Histologic examinations of the tumors in the control and CCl_4 groups revealed mostly HCC (Edmondson grade II) (Fig. 5A). Most HCCs were positive for γ-GTP activity (Fig. 5B), but some areas were negative (Fig. 5C), even within the same tumor tissue. None of the rats showed remote extrahepatic metastases. Serum γ-GTP activities were markedly elevated in HCC-bearing rats as shown in Table 1. Slight serum AFP elevations were observed at the 8th week

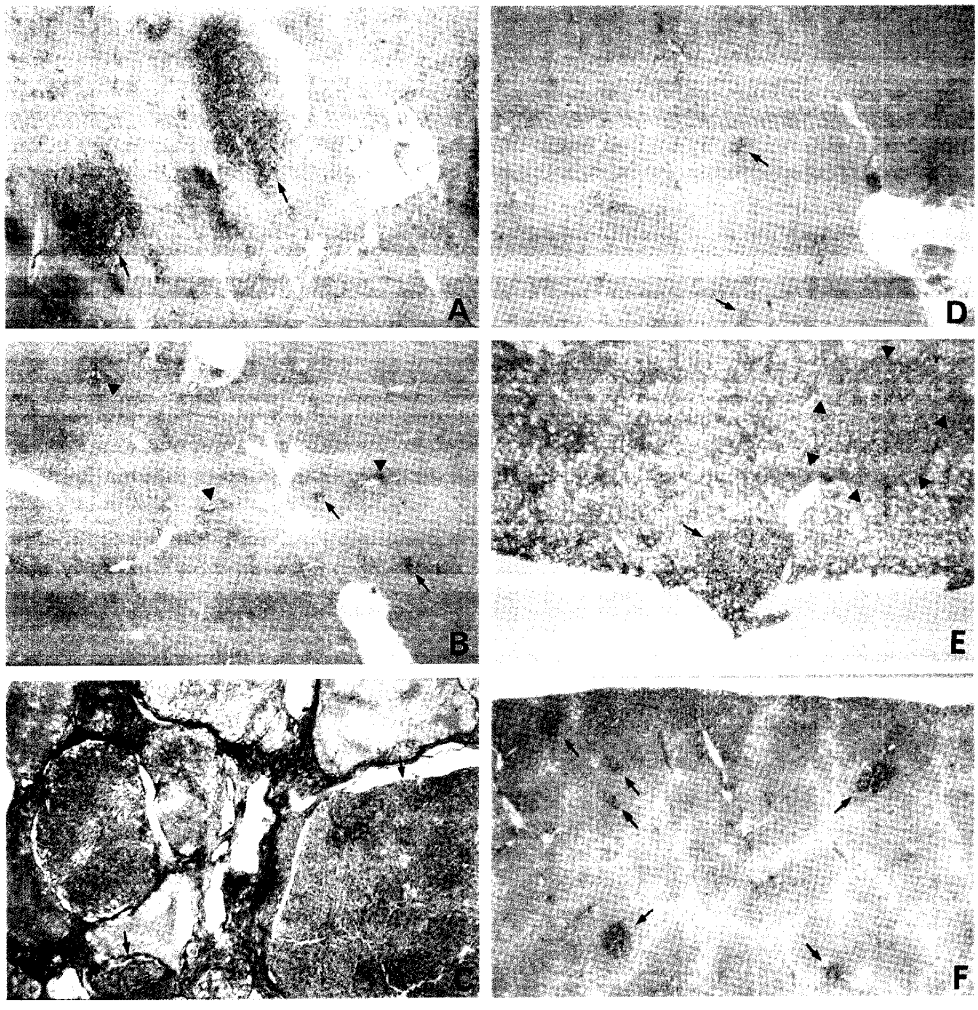

Fig. 3. γ-GTP-positive hyperplastic liver nodules and foci which developed in the 8th and 12th week of Experiments A and B, respectively. ×25 (A) Large γ-GTP-positive foci (arrows) in the control group of Experiment A. (B) γ-GTP-positive area around the bile ducts (arrow heads) and small foci (arrows) in the AZP group of Experiment A. (C) Large hyperplastic liver nodules (arrows) in the CCl_4 group of Experiment A. (D) Small γ-GTP-positive foci (arrows) in the control group of Experiment B. (E) Fatty-infiltrated large foci (arrow and arrow head) in the CD group of Experiment B. (F) Round foci (arrows) in the CCl_4 group of Experiment B.

in all the groups, and marked elevations were seen at the latter stage of the experiment in the control and CCl_4 groups, but low levels in the AZP group (Fig. 6).

Differences in the biochemical characteristics of hyperplastic liver nodules and HCC in each group were evaluated by determining the BCAA-TAase isozyme pattern, and liver AH and ODC activities. Hyperplastic liver nodules in the con-

TABLE 2. AREA AND NUMBER OF γ-GTP-POSITIVE HYPERPLASTIC NODULES AND FOCI OF THE LIVER AND INCIDENCE OF HEPATOCELLULAR CARCINOMA IN RATS TREATED WITH DEN AND FAA

Group	Hyperplastic nodules and foci (8th wk)		Hepatocellular caricinoma (21st - 36th wk)
	Total area (mm^2/cm^2)	Number ($/cm^2$)	No. of tumor bearing rats/ Total rats
Control	31±3	76±7	2/6
AZP	6±3**	20±7**	0/5
CCl_4	56±2**	31±3*	4/5

*$p < 0.01$ **$p < 0.001$

trol and CCl_4 groups contained isozymes I and III and also a small amount of isozyme II (data not shown). However, isozyme III was not observed in hyperplastic liver nodules of the AZP group. The appearance of isozyme III and the decrease in isozyme II were marked in HCC from both the control and CCl_4 groups, and quantitative differences between the two groups were not observed. ODC and AH activities throughout the experiment are summarized in Table 3. The ODC activity in hyperplastic liver nodules of the CCl_4 group was much higher than that of the other groups. However, there was no difference in ODC activity in HCC of the control and CCl_4 groups. Low AH activity was detected in HCC of the control and CCl_4 groups. Peripheral lymphocyte counts in the AZP group decreased to 48 ± 2% (not treated: 73 ± 4, $p<0.01$), and the spleen weight was high (0.23 ± 0.04) as indicated by the ratio of spleen weight to body weight (not treated: 0.18 ± 0.02, $p < 0.05$).

Experiment B. Body weights of animals gradually increased in the CCl_4 and PB groups, but only a slight gain was observed in the CD and AZP groups (Fig. 2B). Peritoneoscopic examination 12 weeks after the beginning of the experiment showed that the liver surfaces in each group were almost smooth without visible nodules (photographs not shown). Elevation of serum GPT activity was minor in all groups, except for the CCl_4 group in which it was slightly high (CCl_4 83 ± 26 IU: Control 51 ± 16). Serum γ-GTP activities were within the normal range in all groups. Histological examination of the liver in H-E staining showed no marked morphological alteration except fatty changes in the CD and CCl_4 groups. However, γ-GTP-positive foci were revealed (Fig. 3). Foci in the CD and CCl_4 groups were consistently and severely affected by fatty infiltration (Fig. 3, E and F). γ-GTP-positive foci in the AZP group resembled those detected in rats fed the FAA- and AZP-mixed diet as observed in Experiment A (Fig. 3B). The area of γ-GTP-positive foci determined in the 12th week was significantly larger in the CD, CCl_4 and PB groups than in the control group (Table 4). However, the area in the AZP group was almost equal to that in the control group. Therefore, no enhancing or inhibitory effect of AZP on foci development was observed in this study.

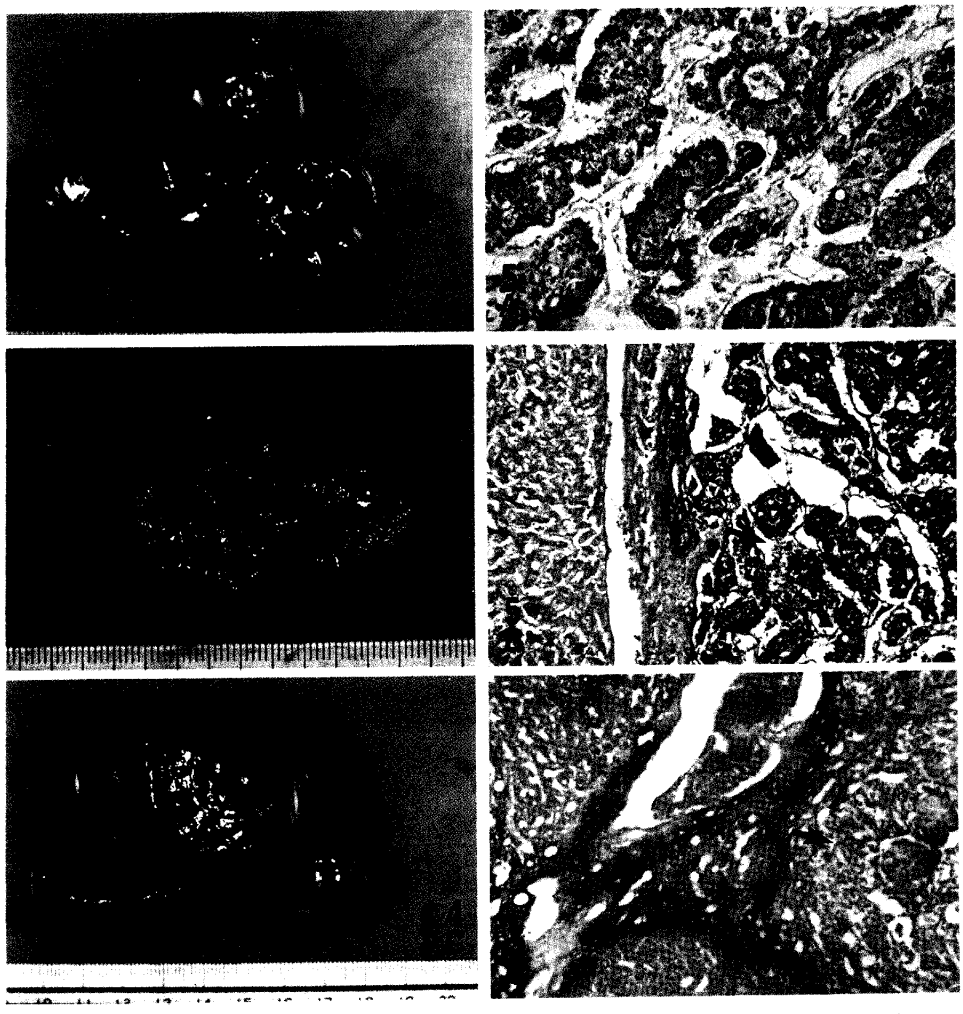

Fig. 4. Gross appearance of livers obtained at the 36th week of Experiment A. (A) A tumor with a smooth white capsule in the control group. (B) Mildly granular dorsal surface of a liver without a tumor in the AZP group. (C) Large tumor in the right lateral lobe in the CCl_4 group.

Fig. 5. Histological and histochemical features of liver tumors from the CCl_4 group obtained in Experiment A. (A) Hepatocellular carcinoma (Edmondson grade II) in H-E stained specimen. × 100 γ-GTP-positive (B, arrow) and negative tissue (C, arrow) of HCC within the same tumor tissue.

Serum γ-GTP levels revealed no correlation between the presence of γ-GTP-positive foci in the liver and serum γ-GTP activity.

Liver AH and ODC activities were also determined to evaluate the differences in the biochemical characteristics of foci in each group which developed in the 12th week of the experiment. ODC activity in the liver of the CD (78 ± 14 pmoles/20 min/mg protein) and CCl_4 (69 ± 5) groups was much higher than that of the

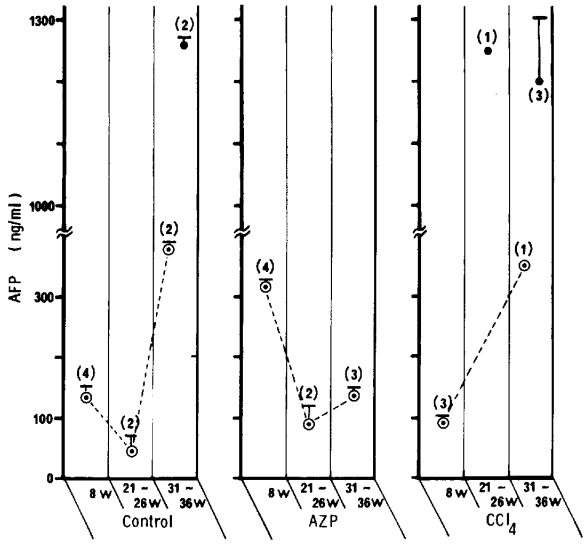

Fig. 6. Serum AFP levels in different periods of Experiment A. Changes in serum AFP levels in rats without HCC are indicated by dotted lines. ●, HCC-bearing rats. ⊙, rats without HCC. ( ): Number of rats examined. Vertical lines indicate standard deviation of the mean.

TABLE 3. LIVER ANILINE HYDROXYLASE AND ORNITHINE DECARBOXYLASE ACTIVITIES IN DEN- AND FAA-TREATED RATS

Group	Week of the exp.	No. of rats	AH (μU/mg protein)	ODC (pmoles/20 min/mg protein)
Normal		(3)	117± 9	3±0.1
Control				
HN	[8]	(3)	57±13	24±2
Non-cancer liver	[21-36]	(4)	42±8	9±2
Cancer liver	[31-36]			
Non-tumor		(2)	35	13
Tumor		(2)	<3	56
AZP				
HN	[8]	(3)	70±16	28±3
Non-cancer liver	[21-36]	(5)	51± 2	13±2
CCl_4				
HN	[8]	(3)	29±15	73±9
Non-cancer liver	[31-36]	(1)	30	11
Cancer liver	[21-36]			
Non-tumor		(4)	36± 9	13±2
Tumor		(4)	<3	68±3

HN = Hyperplastic liver nodule
*$p < 0.01$ **$p < 0.001$

TABLE 4. AREA AND NUMBER OF γ-GTP-POSITIVE FOCI IN RAT LIVER WITHOUT THE USE OF FAA

Group	Hyperplastic foci	
	Total area (mm^2/cm^2)	Number ($/cm^2$)
Control	0.075±0.042	4.4±2.2
AZP	0.055±0.021	2.9±1.0
PB	0.874±0.287**	19.5±2.8***
CCl_4	1.077±0.028****	20.3±1.8***
CD	4.863±0.639****	13.5±3.6*

*$p < 0.05$ **$p < 0.02$ ***$p < 0.01$ ****$p < 0.001$
Number of rats examined = 3

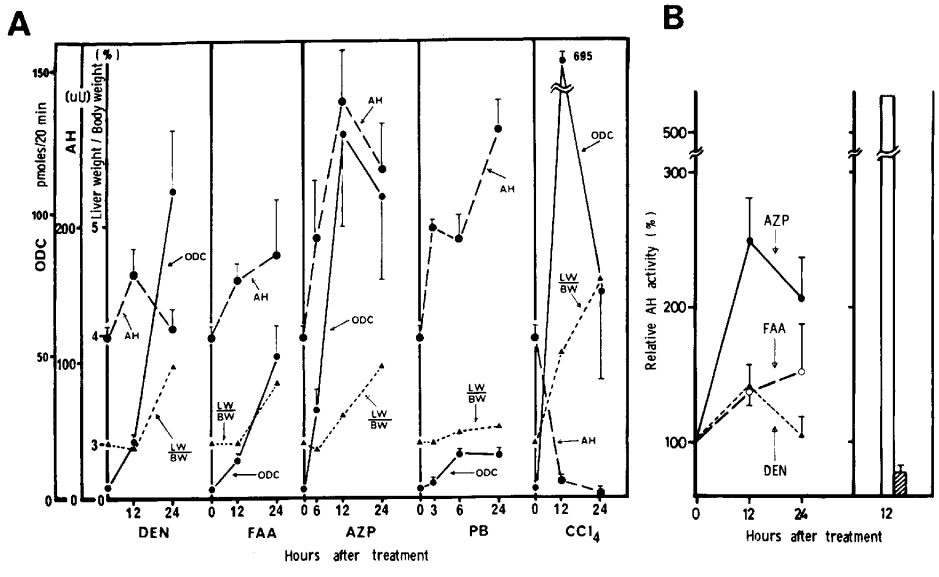

Fig. 7. Alteration of hepatic ODC and AH activities and percent liver weight 3 to 24 h after a single dose of various agents (DEN, FAA, AZP, PB and CCl_4) (A). Percent liver weight (LW/BW) was calculated as (liver weight/body weight) × 100. ●——●, ODC; ●- - -●, AH, and ▲····▲, LW/BW. Relative AH activity was calculated from the results of Experiment C by dividing AH activity at the 12th and 24th h of the various treatments by those before treatment (B). ▲····▲, DEN; ○- - -○, FAA, and ●——●, AZP. The open bar on the right indicates the theoretical relative AH activities calculated by adding each relative AH activity shown on the left in DEN-, FAA- and AZP-treated rats. The hatched bar indicates the measured activities obtained from Experiment D.

control group (25 ± 3). Liver AH activity was higher in the PB group (339 ± 49 uU/mg protein), but less in the CCl_4 group (61 ± 4, $p < 0.01$) than in the control group (102 ± 1, $p < 0.001$). There did not appear to be any quantitative differences in the ODC and AH activities of the hyperplastic liver nodules and foci between Experiment A and Experiment B.

Experiment C. Administration of AZP and other chemicals used in both Experiments A and B resulted in a marked increase in hepatic ODC activity as early as 3 to 12 h after the treatment, and the maximal ODC activity was observed within 24 h of the treatment (Fig. 7A). Increasing or decreasing activity of liver AH was proportional to ODC activity except in the case of CCl_4 administration. Liver weights also increased in proportion to the elevation of ODC activity. PB caused a slight but significant increase in ODC activity, followed by increasing AH activity and liver weight. The data suggests that AZP, CCl_4 and other chemicals induce hepatic ODC activity, and that subsequent AH induction and liver hypertrophy might act as a promoter of hepatocarcinogenesis.

Experiment D. The increased activity of liver AH observed 12 h after a single dose of FAA, AZP or DEN was not observed when AZP was administered simultaneously with FAA to rats treated with DEN beforehand (Fig. 7B).

DISCUSSION

Increased γ-GPT-positive hyperplastic liver nodules and a very high incidence of HCC in DEN-treated rats fed a diet containing FAA were observed following additional repeated injections of CCl_4. The enhancing effect of CCl_4 on the evolution of hyperplastic liver nodules and HCC may be due to the combination of CCl_4 and FAA having a synergistic effect. CCl_4-induced liver injury possibly results from free radical attack of the cell membrane causing hepatocellular necrosis and regeneration (18). CCl_4 might make necrosis and regeneration more intense when rats are treated with FAA. A reasonable interpretation of the enhanced hyperplastic liver nodules and HCC production in the CCl_4 group is that the repeated doses of CCl_4, leading to an enhancement of hepatocyte regeneration, promoted DEN- and FAA-initiated cells during hepatocarcinogenesis.

Even without exposure to FAA, additional doses of CCl_4 or a CD diet also effectively enhanced the evolution of γ-GTP-positive foci as observed in the case of PB administration. Feeding of a CD diet has been reported to induce a number of functional and structural alterations of the membranous organelles of hepatocytes (19) and cause low grade liver damage (3). Since regeneration follows the appearance of widely scattered liver cell necrosis, the CD diet has a mitogenic action as strong as that of a partial hepatectomy and accelerates the evolution of chemical carcinogen-initiated cells, which have been shown to progress to γ-GTP-positive foci (3). The enhancing effect of CCl_4 in rats injected with DEN alone was also thought to occur from injured liver cells being susceptible to carcinogenic action and providing the stimulus for DEN-initiated cell evolution.

Since both liver hypertrophy and induction of microsomal mixed-function oxygenases, which result from increased hepatic ODC activity (13), have been suggested as being essential for tumor promotion, and prolonged elevation of ODC activity has been reported to be necessary for hepatocarcinogenesis (20), the promoting activity of CCl_4 and CD diet may be evaluated by the inducibility of he-

patic ODC activity. Marked elevation of hepatic ODC activity and increased liver weight were observed after a single dose of CCl_4, and hepatic ODC activity in hyperplastic liver nodules and foci was very high in rats treated with CCl_4 or fed on a CD diet.

The most outstanding result of the present study was the apparent AZP suppression of hyperplastic liver nodules and HCC development. Immunosuppresive agents are generally recognized to promote the development of tumors induced by chemical carcinogens (21). Neoplasms and their metastases appear to proliferate when organ-transplanted patients are on immunosuppressive therapy (5). However, the relation of immunosuppression to the induction of primary tumors is not fully understood at the present time (5).

AZP has been reported to induce liver cell necrosis (22) and impaired liver cell regeneration (23). AZP administration also induced a much more severe fibrosis and cirrhosis in CCl_4-treated rats (24). The dose of AZP used in the present study is hepatotoxic according to a previous experiment (22). A single hepatotoxic dose of AZP produces a marked increase in hepatic ODC activity, followed by an increase in liver weight. Since the promoting action of CCl_4 was associated with markedly elevated ODC activity and increased liver weight, AZP should act as a promoter of hepatocarcinogenesis. However, AZP apparently suppressed hepatocarcinogenesis in DEN- and FAA-treated rats, and no enhancing effect was observed in DEN-injected rats without exposure to FAA. There has been no experimental or clinical evidence presented that AZP has a suppressive effect on hepatocarcinogenesis.

Suppression of hepatocarcinogenesis by AZP might be due to the altering of FAA activation. It has been reported that the hepatic necrosis produced by N-acetylarylamine is prevented by prior treatment with inhibitors of cytochrome P-450 (25). Therefore, the depression of mixed-function oxidase activities in the liver, particularly those concerned with the metabolism of FAA, may play a role in the suppressive effect of AZP on hepatocarcinogenesis. Liver AH activity increased 12 h after a single intragastric dose of FAA or AZP or intraperitoneal injection of DEN, but was greatly reduced by the simultaneous administration of FAA and AZP in rats treated with DEN in advance. The mechanisms of the inhibitory effect on the evolution of hyperplastic liver nodules and HCC observed in DEN-treated rats with simultaneous administration of FAA and AZP could be explained by the interaction between AZP and FAA. N-Hydroxylation of N-acetylarylamines is dependent on a cytochrome P-450-dependent mixed-function oxidase in liver microsomes (25). N-Hydroxylation is now recognized as the initial and critical step in the metabolic activation of carcinogenic N-acetylarylamide (26). A reasonable interpretation of the apparent suppression by AZP of hepatocarcinogenesis is that microsomal enzyme activity catalyzing N-hydroxylation of FAA is diminished, and the anabolic process to ultimate carcinogens is not favored. The possibility of an alteration in FAA metabolism being related to the inhibition of

hepatocarcinogenesis is supported by previous reports that the simultaneous administration of methylcholanthrene (27) or phenobarbital (10) suppressed FAA hepatocarcinogenesis. The suppressive effects of these two microsomal function-affecting drugs apparently result from induction of mixed-function oxidase activity, shifting the balance of FAA metabolism toward the detoxification pathways.

Another possible mechanism of the suppressive effect of AZP on the evolution of hyperplastic liver nodules and HCC might be the inhibition of liver cell regeneration (22) as judged by liver weight, since they were much lower in DEN-treated rats fed on the diet containing FAA and AZP. AZP may also inhibit liver fibrogenesis in the process of hepatocarcinogenesis. Inhibition of DEN-induced hepatocarcinogenesis by aminoacetonitrile, a lathyrogenic agent, has been reported (28). AZP depression of CCl_4-induced fibrogenesis (29) is indicated by the fact that the hydroxyproline content in the livers of rats given CCl_4 plus AZP was significantly lower than in rats given CCl_4 alone. However, Experiments A and B of the present study show no indication of decreased fibrogenesis by AZP administration.

Suppression by AZP of DEN-induced hepatocarcinogenesis without the use of FAA was not clearly shown in Experiment B, because the hepatocarcinogenic action of a single dose of DEN appeared to be relatively weak and to result in only slight foci development in the liver. The results were almost the same as those obtained with DEN-treated rats fed on the AZP-containing diet. However, the present study did demonstrate that AZP at least does not promote the development of hyperplastic liver nodules and foci, and HCC.

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